Journal of Infection and Public Health (2015) 8, 619—625



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# Molecular analysis of *rpoB* gene mutations in rifampicin resistant *Mycobacterium tuberculosis* isolates by multiple allele specific polymerase chain reaction in Puducherry, South India

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Received 13 February 2015; accepted 16 May 2015

**KEYWORDS**
*Mycobacterium tuberculosis*;
Rifampicin;
Multiple allele-specific PCR;
Drug resistance;
*rpoB* gene

**Summary**
*Background:* *rpoB* gene mutations in *Mycobacterium tuberculosis* (MTB) make the bacteria resistant to rifampicin. Thus, these mutations are surrogate markers for multi-drug resistance (MDR). The objective of this study was to evaluate an allele-specific multiplex-polymerase chain reaction (MAS-PCR) assay to detect mutations at codons 516, 526 and 531 of the *rpoB* gene.
*Methods:* In total, 127 *M. tuberculosis* clinical isolates were subjected to standard drug susceptibility tests. A MAS-PCR assay was then performed to detect mutations in the *rpoB* gene. Three different allele-specific PCR assays were performed (single-step MAS-PCR) and the amplified products were sequenced.
*Results:* Of the 127 isolates, 69 (54.3%) were multidrug resistant *M. tuberculosis* (MDR-TB), 21 (16.5%) were rifampicin mono-resistant and 37 (29.1%) were drug susceptible. The frequency of mutations at codons 531, 526 and 516 was 54.4%, 18.9% and 5.6%, respectively. A triple mutation was found in 4 (4.4%) isolates. Mutations

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http://dx.doi.org/10.1016/j.jiph.2015.05.003

in regions other than the 81-bp region were observed at codons 413 (11.1%), 511 (12.2%) and 521 (15.6%) of the *rpoB* gene.
*Conclusions:* The simplicity and specificity of the MAS-PCR assay allows for easy implementation in clinical laboratories to detect rifampicin drug resistance in MDR-TB strains.


## Introduction

Worldwide, there are an estimated 9 million new cases and 1.4 million deaths caused by *Mycobacterium tuberculosis* (MTB) infections [1]. These numbers are increasing due to the increasing emergence and spread of multi-drug resistant *M. tuberculosis* strains (MDR-TB). MDR-TB strains are now a serious threat to public health control systems and do not have effective treatment regimens [2]. Rifampicin (RIF) and isoniazid (INH) are the drugs of choice to treat MTB infections [3]. However, organisms have started showing resistance to these drugs. RIF resistance ($RIF^r$) is caused by mutations in a 81-bp region (codons 507—533, 27 amino acids) of the *rpoB* gene [4]. Therefore, this 81-bp region is called the rifampicin resistance determining region (RRDR) (RNA polymerase enzyme binding site) [3—6]. It is imperative to identify rifampicin resistant strains and prevent the spread of MDR-TB [6,7]. Cost-effective, sensitive PCR-based techniques followed by DNA-sequencing of targeted individual codons are used to determine RIF resistance [8,9]. One such PCR technique is multiplex allele-specific PCR (MAS-PCR), which detects $RIF^r$. MAS-PCR utilizes three different specific primers to detect mutations at codons 516, 526 and 531. These mutations are responsible for drug resistance in the majority of RIF resistant-strains (70 to 95%) [10—12].

Therefore, this study aimed to evaluate a rapid detection method of common mutations in the RRDR in the *rpoB* gene of $RIF^r$ MTB clinical isolates using a MAS-PCR assay followed by DNA sequencing.

## Materials and methods

### Study settings

In total, 127 samples were received from TB-suspected patients (both in-patient and out-patient) between January 2011 and July 2013. Samples were collected from out-patients based on clinical symptoms, smear results and chest X-ray analyses that were positive for TB and from hospitalized patients based on a physician's recommendation.

### Bacterial strains and drug susceptibility testing

Species identification was performed using conventional mycobacteriological methods, such as colony morphology in Lowenstein—Jensen (LJ) medium, acid-fast staining (AFB), and biochemical tests [13]. Sputum samples were processed and used for inoculation on LJ slants and DNA isolation. Tests were performed to differentiate the MTB complex from non-tuberculous mycobacteria (NTM) in clinical specimens.

A drug susceptibility test (DST) was performed with 40 μg/mL of rifampicin to identify $RIF^r$ [14]. *M. tuberculosis* H37Rv (ATCC 27294), standard laboratory drug susceptible and MDR-TB strains were used as controls.

### Extraction of mycobacterial DNA

MTB clinical isolates were subjected to genomic DNA extraction following a phenol: chloroform extraction method [15]. Isolated genomic DNA was refrigerated at 4 °C for further use.

### PCR procedures

Amplification of IS*6110* (123-bp) using primers IS1 and IS2 for species level mycobacterium identification (Table 1) was performed as described previously [1]. Amplification of the *rpoB* gene in both $RIF^s$ and $RIF^r$ isolates was performed with the primers *rpoB95* and *rpoB397* [16] (Table 1), which generated a 329-bp amplified product that was used for DNA sequencing

#### Multiplex allele-specific PCR (MAS-PCR)

MAS-PCR was performed using three allele-specific primers targeting mutated codons 516, 526, and 531 of the *rpoB* gene as previously described [11].

**Table 1** MAS-PCR Primers and DNA sequencing primers to detect RIF resistance mutations of *M. tuberculosis* clinical isolates.

| Target gene | Primer/alleles | Primer sequences (5′—3′) | PCR product length (bp) |
|---|---|---|---|
| IS*6110*[a] | IS1 | GTGAGGGCATCGAGGTGG | 123 |
| | IS2 | CGTAGGCGTCGGTCACAAA | |
| *rpoB* | *rpoB* 516 (+) | CAGCTGAGCCAATTCATGGA | 218 |
| | RIRm (−) | TTGACCCGCGCGTACAC | |
| | *rpoB* 526 (+) | CTGTCGGGGTTGACCCA | 185 |
| | RIRm (−) | TTGACCCGCGCGTACAC | |
| | *rpoB* 531 (+) | CACAAGCGCCGACTGTC | 170 |
| | RIRm (−) | TTGACCCGCGCGTACAC | |
| *rpoB* RRDR[b] | *rpoB* 95 | CCACCCAGGACGTGGAGGCGATCACAC | 329 |
| | *rpoB* 397 | CGTTTCGATGAACCCGAACGGGTTGAC | |

[a] For species identification and routine TB diagnosis.
[b] The amplified product containing RRDR region of *rpoB* gene for DNA sequencing.

The complete list of primers used in this study is listed in Table 1. The MAS-PCR primers bind specifically to wild-type sequences of the *rpoB* gene (codons 516, 526, and 531). If a mutated allele is present, there will not be an amplification, which results in the absence of the corresponding band on an agarose gel [11].

### DNA sequencing analysis

The PCR-amplified fragment (329-bp) of the *rpoB* gene containing RRDR (81-bp) was subjected to direct DNA sequencing (Indian Institute of Technology (IIT), Chennai, India) to confirm the results from the MAS-PCR assay and identify mutations at specific bases in codons 516, 526 and 531 of the *rpoB* gene and other regions.

### Statistical analysis

The sensitivity and specificity of the MAS-PCR assay was calculated based on the detection of $RIF^r$ in clinical isolates using a DNA sequencing method as the gold standard and a conventional drug susceptibility test as a control. MedCalc (version 12.5) (MedCalc Software, Ostend, Belgium) was used to calculate and interpret the test parameters for sensitivity and specificity [17].

## Results

### Conventional assays

Colony morphology, biochemical tests and PCR amplification of IS*6110* were performed on all of the isolates from confirmed MTB infections (other routine investigations are not illustrated in this paper).

### Drug susceptibility testing

Of the 127 *M. tuberculosis* clinical isolates, 69 (54.3%) were MDR-TB, 21 (16.5%) were RIF mono resistant and 37 (29.1%) were drug susceptible against specific anti-TB drugs.

### MAS-PCR assay

MAS-PCR was performed. After amplification, the PCR products were subjected to agarose gel electrophoresis (Fig. 1). The $RIF^r$ phenotype is classified by the absence of (one or more) the corresponding bands in the isolates: 218-bp (Fig. 1a), 185-bp (Fig. 1b), and 170-bp (Fig. 1c). $RIF^s$ isolates showed bands in the gel. A concordant result was obtained by DNA sequencing the RRDR of all of the isolates included in this study. Isolates susceptible to RIF produced amplified products. Isolates resistant to RIF failed to produce amplified products. The most frequent mutation was observed at codon 531 of the *rpoB* gene in 68 (75.5%) strains, followed by a mutation at codon 526 in 17 (18.9%) strains and a mutation at codon 516 in 5 (5.6%) strains. A mutation at codon 531 was combined with 526 codon mutations in 15 strains (16.7%), and only 4 isolates (4.4%) showed triple mutation at codons 516, 526 and 531 (Table 2). All of the susceptible isolates contained wild-type sequences. The results of the *in vitro* conventional drug susceptibility tests revealed susceptible patterns for the susceptible isolates.

### DNA sequencing

The conventional solid DST and MAS-PCR assay results were further compared with the amplified products of the *rpoB* gene (329 bp) containing RRDR using automated DNA sequencing. The frequencies

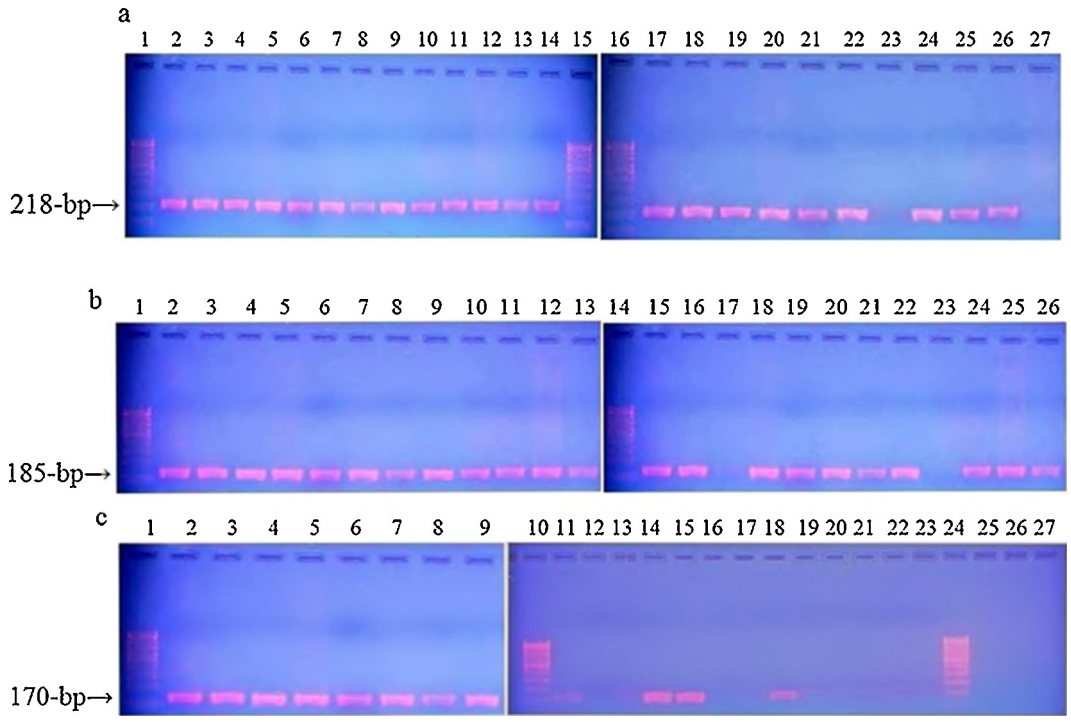


**Figure 1** Examples of banding profiles generated by MAS-PCR with purified DNA preparations from *M. tuberculosis* clinical isolates by targeting *rpoB* codons: codon 531 (1a), codon 526 (1b), and codon 516 (1c). In general, the presence of the bands corresponded to size: codon 516 at 218 bp, codon 526 at 185 bp and codon 531 at 170 bp represent the presence of the wild type allele and RIF susceptible strains. The absence of these bands indicates the mutated alleles of RIF resistance strains. (a) Lanes 1, 15 and 16: 100-bp DNA Ladder (HiMedia, India), Lane: 2, positive control *M. tuberculosis* H37Rv strain; Lanes: 3—14 and 17—27, 218-bp product of strains with *rpoB* 516 mutant alleles (GTC, TAC, and GGC). (b) Lanes 1, 14: 100-bp DNA Ladder (HiMedia, India), Lane: 2, Positive control *M. tuberculosis* H37Rv; Lanes 3—13 and 15—26, 185-bp product of strains with *rpoB* 526 mutant alleles (GAC, TAC, and CTC). (c) Lanes 1, 10 and 24: 100-bp DNA Ladder (HiMedia, India), Lane: 2, Positive control *M. tuberculosis* H37Rv; Lanes 3—9, 11 to 23 and 25—27, 170-bp product of strains with *rpoB* 531 mutant alleles (TCG and TTG).

of the mutations identified in the 127 isolates are shown in Tables 2 and 3. Mutations in regions other than the RRDR were observed and widely distributed among the RIF$^r$ strains. They were also observed in the drug-resistant phenotypes using *in vitro* DST.

In total, three strains (3.3%) of MDR-TB failed to amplify using the MAS-PCR assay, but their sequences were determined by DNA sequencing. In total, mutations in regions other than the 81-bp region of the *rpoB* gene were observed in 73 (57.5%) isolates (Table 3). Mutations at codons 413 (11.1%), 511 (12.2%) and 521 (15.6%) were frequently observed. The mutations detected by MAS-PCR and DNA sequencing are listed in Tables 2 and 3.

## Statistical analysis: sensitivity and specificity

Of the total 127 isolates, 68 of the 69 MDR-TB isolates, 19 of the 21 RIF$^r$ (rifampicin mono resistant) isolates and 36 of the 37 susceptible isolates were

**Table 2** Common mutations in RRDR region detected by MAS-PCR assay associated with RIF-resistance and their frequency.

| Gene | Rifampicin | | | | |
|---|---|---|---|---|---|
| | *rpoB* | | | | |
| Mutations | D516V | H526Y | S531L | H526Y + S531L | D516V + H526Y + S531L |
| No. of strains | 5 | 17 | 49 | 15 | 4 |
| Frequency (%) | 5.6 | 18.9 | 54.4 | 16.7 | 4.4 |

**Table 3** Frequency of other mutations found in *rpoB* gene among RIF resistant *M. tuberculosis* isolates.

| Codon no | Nucleotide changes | Amino acid changes | No. of strains (%) |
|---|---|---|---|
| 413 | AAC → CAC | Asn → His | 10 (11.1) |
| 435 | GAC → GAG | Asp → Glu | 6 (6.7) |
| 451 | GCA → GAC | Ala → Asp | 8 (8.9) |
| 511 | CGC → TGC | Arg → Cys | 11 (12.2) |
| 513 | GTC → GAC | Val → Asp | 9 (10) |
| 521 | GAG → GAC | Glu → Asp | 14 (15.6) |
| 451 + 511 | GCA → GAC, CGC → TGC | Ala → Asp, Arg → Cys | 7 (7.8) |
| 513 + 521 | GTC → GAC, GAG → GAC | Val → Asp, Glu → Asp | 8 (8.9) |

successfully identified using the MAS-PCR assay. The sensitivity of MAS-PCR to detect drug resistance in clinical isolates (both MDR-TB and RIF$^r$ isolates) was 96.7%, and the specificity was 97.3%.

## Discussion

Early detection of drug resistance in MTB allows for a timely implementation of a treatment regimen that can better control the infection [1,12,18,19]. During the last few decades, several approaches have been proposed for the rapid detection of MDR-TB in a clinical setting, including both genotypic and phenotypic methods [11,12,15,16,19,20]. In almost all cases, molecular methods are the default for detecting RIF drug resistance because it is considered to be a surrogate marker for MDR-TB [15,16,19,20]. The advantage of a genotypic method is that it requires a shorter time than other routine methods. However, not all of the molecular methods for detecting drug resistance are low-cost. Therefore, they are not routinely implemented in all clinical mycobacteriology laboratories [4,21].

Prior to the validation and optimization of MAS-PCR, routine molecular diagnostic assays, such as the IS*6110* and *mtp40* PCR assays, were performed on DNA samples of MTB clinical isolates: (i) IS*6110*-PCR, a routine diagnosis of TB infection [1], and (ii) *mtp40*-PCR, for species identification of MTB from NTM isolates [1]. Consequently, MAS-PCR analysis was performed on purified genomic DNA samples from 90 RIF$^r$ isolates and 37 RIF$^s$ isolates from patients enrolled from 2011 to 2013.

The MAS-PCR assay generated banding profiles for different mutations in the *rpoB* gene (codons 516, 526, and 531) that were successfully identified by the respective codon-specific primer PCR (Fig. 1). The stringency of allele-specific PCR has been standardized. PCR assays targeting specific point mutations were performed in duplicate or triplicate. Therefore, MAS-PCR successfully identified the *rpoB*516, *rpoB*526 and *rpoB*531 mutations in clinical isolates. The most prevalent mutations affected codon 531 (75.5%), followed by codon 526 (18.9%) and codon 516 (5.6%). Among the drug resistant isolates, mutations at codon 531 occurred alone in 49 (54.4%) cases. A combination of mutations was also observed as a double mutation associating codons 526 and 531 in 15 (16.7%) strains and a triple mutation at codons 516, 526 and 531 in 4 (4.4%) strains. Several supporting studies showed that the occurrence of a frequent mutation at S531L is the most predominant worldwide [3,11,12,15,16,19,20,22–24].

The *rpoB* 329-bp amplified product of all of the RIF$^s$ and RIF$^r$ isolates in the RRDR was sequenced. The mutations in the RRDR were identified, and the results were analyzed in parallel with the MAS-PCR and conventional drug susceptibility results. The conventional solid LJ system exhibited the same result patterns. The extent of drug resistance was also analyzed. Of the 90 RIF$^r$ isolates, 7 demonstrated a level of resistance against RIF at a high MIC (>128 mg/L). However, other RIF$^r$ isolates showed resistance at 40 μg/ml. There was no correlation between the presence of a particular codon mutation and the level of drug resistance in the clinical isolates. Almost all of the isolates had a 531 codon mutation.

The conventional assay failed to detect a drug resistance phonotype in 5 (3.9%) strains due to a low bacilli load. Genomic DNA was used to acquire a resistance pattern using molecular methods. The MAS-PCR method failed to identify drug resistance in two strains (2.2%). In this study unique mutations in the RRDR at codons 511 and 521 were frequently found in 11 (12.2%) and 14 (15.6%) isolates, respectively (Table 3). Of the 21 RIF$^r$ isolates, 2 (9.5%) showed no bands from the MAS-PCR assay because of the presence of other codon mutations. Several studies were performed worldwide and reported occurrences of other mutations in and out of the 81-bp RRDR [19,20,23–25]. The presence of mutations outside the 81-bp region have rarely been reported. They were reported in less than 5% of RIF$^r$ strains in

**Table 4** Diagnostic indexes of MAS-PCR assay at 95% Confidence Interval (CI).

| MAS-PCR | LJ culture | | | Sens (%) | Spec (%) | PPV[d] (%) | NPV[e] (%) | LR (+)[f] | LR (−)[g] |
|---|---|---|---|---|---|---|---|---|---|
| | R[a] | S[b] | NVC[c] | | | | | | |
| **MDR-TB** | | | | | | | | | |
| Detection | 68 | 0 | 1 | | | | | | |
| No detection | 0 | 0 | 0 | | | | | | |
| **RIF mono-resistance** | | | | | | | | | |
| Detection | 16 | 0 | 3 | 96.7 (90.55–99.27) | 97.3 (85.79–99.55) | 98.9 (93.81–99.81) | 92.3 (79.11–98.30) | 35.8 (5.17–247.33) | 0.03 (0.01–0.10) |
| No detection | 2 | 0 | 0 | | | | | | |
| **Drug susceptible** | | | | | | | | | |
| Detection | 1 | 0 | 1 | | | | | | |
| No detection | 0 | 35 | 0 | | | | | | |

Sen = sensitivity; Spe = specificity.

[a] R = resistant.

[b] S = Sensitive.

[c] NVC = no visible colony.

[d] PPV = positive predictive value.

[e] NPV = negative predictive value.

[f] LR(+) = positive likelihood ratio.

[g] LR(−) = negative likelihood ratio.

DNA sequencing studies [3,20]. The positive predictive value of MAS-PCR was 98.9%, and the negative predictive value was 92.3% (Table 4). Although, a sequencing method is a costly and laborious process to confirm results, it can be incorporated to diagnose drug resistant isolates with high specificity and efficiency. Thus, it is needed to detect drug resistance in isolates due to rare, clinically important mutations.

## Conclusions

In conclusion, MAS-PCR was performed to detect common mutations of the *rpoB* gene. It is a simple and rapid molecular method to identify RIF drug resistance in *M. tuberculosis* clinical isolates. More importantly, the interpretation of the MAS-PCR results is easier than other methods. It is inexpensive and requires fewer resources to set up. It can also be implemented with routine clinical resources in laboratories in resource-poor settings.

## Conflicts of interest

The authors declare no conflict of interest.

## Acknowledgements

We thank Mr. G. Stalin Chellappa for providing technical support and for helping to prepare this manuscript.

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